09/582779

529 Rec'd PCT/PTO 03 JUL 2000

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER 48715

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP 98/08382	~~15 January 1998~~ 18 DEC 1998	15 January 1998

TITLE OF INVENTION: OROTIDINE-5'-PHOSPHATE DECARBOXYLASE GENE, GENE CONSTRUCT COMPRISING THIS GENE AND ITS USE

APPLICANT(S) FOR DO/EO/US Markus POMPEJUS, Jose Luis Revuelta DOVAL, Maria Angeles Santos GARCIA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. /X/ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. / / This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. /X/ This express request to begin national examination procedures (35 U.S.C.371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. /x / A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. /X/ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).

   a./X/ is transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / has been transmitted by the International Bureau.
   c./ / is not required, as the application was filed in the United States Receiving Office (RO/US0).

6. /X/ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. /X/ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).

   a./X/ are transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / have been transmitted by the International Bureau.
   c./ / have not been made; however, the time limit for making such amendments has NOT expired.
   d./ / have not been made and will not be made.

8. /X/ A translation of the amendments to the claims under PCT Article 19(35 U.S.C. 371(c)(3)).

9. /X/ An oath or declaration of the inventor(s)(35 U.S.C. 171(c)(4)).

10./ / A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11./X/ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12./X/ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13./X/ A FIRST preliminary amendment.
/ / A SECOND or SUBSEQUENT preliminary amendment.

14./ / A substitute specification.

15./ / A change of power of attorney and/or address letter.

16./x / Other items or information.
International Search Report
International Preliminary Examination Report

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U.S. Appln. No. (If Known)	INTERNATIONAL APPLN. NO.	ATTORNEY'S DOCKET NO.
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17. /X/ The following fees are submitted	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO...$840.00	840.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482).......................................$750.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).........................$700.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ....$ 970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied pro -visions of PCT Article 33(2)-(4).....................$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT = $	840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than / / 20 / /30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate		
Total Claims	3 -20		X$18.		
Indep.Claims	2 -3		X$78.		
Multiple dependent claim(s)(if applicable)			+270.		
TOTAL OF ABOVE CALCULATION			=	840.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					
SUBTOTAL			=	840.00	
Processing fee of $130. for furnishing the English translation later than / /20 / /30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					
TOTAL NATIONAL FEE			=	840.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property =				40.00	
TOTAL FEES ENCLOSED			= $	880.00	

Amount to be refunded: $______
Charged $______

a./X/ A check in the amount of $ 880. to cover the above fees is enclosed.

b./ / Please charge my Deposit Account No.__________in the amount of $__________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c./X/ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 11-0345. A duplicate copy of this sheetis enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

Herbert B Keil
SIGNATURE

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18,967
Registration No.

09582779

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	
POMPEJUS et al.	)	BOX PCT
	)	
International Application	)	
PCT/EP 98/08382	)	
	)	
Filed: December 18, 1998	)	
	)	

For: OROTIDINE-5'-PHOSPHATE DECARBOXYLASE GENE, GENE CONSTRUCT COMPRISING THIS GENE AND ITS USE

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to examination, kindly amend the above-identified application as follows:

IN THE CLAIMS

Claim 3, line 2, delete "or 2".

Claim 5, line 3, delete "or 2".

Claim 7, lines 1 and 2, delete "or 6.

Claim 8, line 2, delete "or 6".

Claim 9, line 4, delete "or 2".

Claim 10, line 4, delete "or 2".

Claim 12, line 1, delete "or 11".

Claim 13, line 1, delete "any of claims 10 to 12" and insert --claim 10--.

Claim 14, line 2, delete "or 2".

REMARKS

The claims were amended in the preliminary examination. The claims have been amended further to eliminate multiple dependency and to put them in better form for U.S. filing. No new matter is included.

Favorable action is solicited.

Respectfully submitted,

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09/582779

Orotidine-5'-phosphate decarboxylase gene, gene construct comprising this gene and its use

The invention relates to an orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID No. 1 or its homologs, to a gene construct comprising this gene or its homologs, and to its use. The invention additionally relates to vectors or organisms comprising an orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID No. 1 or its homologs.

The invention further relates to a process for producing uracil-auxotrophic microorganisms and to a process for inserting DNA into uracil-auxotrophic microorganisms.

Vitamin B2, also called riboflavin, is essential for humans and animals. Vitamin B2 deficiency is associated with inflammations of the mucosa of the mouth and throat, pruritus and inflammations in skin folds and similar cutaneous lesions, conjunctival inflammations, reduced visual acuity and clouding of the cornea. In babies and children, cessation of growth and weight loss may occur. Vitamin B2 therefore has economic importance in particular as vitamin supplement in cases of vitamin deficiency and as animal feed supplement. It is additionally used as food color, for example in mayonnaise, ice cream, blancmange etc..

Vitamin B2 is prepared either chemically or microbially (see, for example, Kurth et al., 1996, Riboflavin, in: Ullmann's Encyclopedia of industrial chemistry, VCH Weinheim). In the chemical preparation processes, riboflavin is usually obtained as pure final product in multistage processes, it being necessary to employ relatively costly starting materials such as, for example, D-ribose. An alternative to the chemical synthesis of riboflavin is the preparation of this substance by microorganisms. The starting materials used in this case are renewable raw materials such as sugars or vegetable oils. The preparation of riboflavin by fermentation of fungi such as Eremothecium ashbyii or Ashbya gossypii is known (The Merck Index, Windholz et al., eds. Merck & Co., page 1183, 1983), but yeasts such as, for example, Candida, Pichia and Saccharomyces or bacteria such as, for example, Bacillus, clostridia or corynebacteria have also been described as riboflavin producers.

DE 44 20 785 describes six riboflavin biosynthesis genes from Ashbya gossypii, and microorganisms which have been transformed with these genes, and the use of such microorganisms for riboflavin synthesis.

To date, genes have been inserted into fungal riboflavin producers such as Ashbya gossypii via the markers leu2 (leucine auxotrophy), thr4 (threonine auxotrophy) or kan (kanamycin resistance) (WO 92/00379). A further marker described in yeasts is met15 (methionine auxotrophy, Cost et al., Yeast, Vol. 12, 1996: 939 - 941). The disadvantage of this marker is that either the transformation efficiency is very low and/or antibiotics must be continuously added for the selection. However, in each case, counterselection for loss of the marker with retention of the inserted genes in microorganisms is impossible or possible only with very great effort, so that it is usually no longer possible to insert further genes with these markers into the microorganisms. It is therefore desirable to have a selection marker which displays high transformation efficiency, is easily selectable and makes counterselection possible.

The orotidine-5'-phosphate decarboxylase gene (= URA3 gene) from Saccharomyces cerevisiae is one of the classical markers having the required properties and usable for transforming genes into microorganisms such as yeasts and fungi. The isolation of species-specific URA3 genes and the isolation of the corresponding gene from fungi (= pyrG) and the sequences thereof from Pichia stipitis, Candida boidinii, Kluyveromyces marxianus, Yamadazyma ohmeri, Candida maltosa, Aspergillus niger, Aspergillus oryzae, Aspergillus nidulans, Mucor circinelloides, Phycomyces blakesleeanus, Penicillium chrysogenum, and Aspergillus awamori have been described in a number of studies (Appl. Environ. Microbiol., Vol. 60, No. 12, 1994 : 4245 - 4254, Nucl. Acids Res., Vol. 18, No. 23, 1990: 7183, J. Ferment. Bioeng., Vol. 73, No 4, 1992: 255 - 260, Yeast, Vol. 9, 1993: 677 - 681, Yeast, Vol. 10, 1994: 1601 - 1612, Curr. Genet., Vol. 23, 1993: 205 - 210, Nucl. Acids Res., Vol.16, No. 5, 1988: 2339, Curr. Genet., Vol. 16, 1989: 159 - 163, Gene, Vol. 61, 1987: 385 - 399, Gene, Vol. 116, 1992: 59 - 67, Mol. Gen. Genet., Vol. 224, 1990: 269 - 278, Nucl. Acids Res., Vol. 16, No. 16, 1988: 8177, Nucl. Acids Res., Vol. 18, No. 23, 1990: 7183 and Curr. Genet., Vol. 27, 1995: 536 - 540).

Studies by Rose et al. (Gene, Vol. 29, 1984: 113 - 124) have shown that the URA3 gene from Saccharomyces cerevisiae is in fact capable of complementation of a corresponding mutation (pyrF gene = URA3) in prokaryotes such as Escherichia coli, and can be useful as selection marker.

However, genetic studies on riboflavin synthesis by Ashbya gossypii (vitamin B2 synthesis) have shown that the URA3 gene from Saccharomyces cerevisiae or the pyrF gene from Escherichia coli are [sic] not capable of complementation of

uracil-auxotrophic Ashbya gossypii mutants, and therefore these genes cannot be used for cloning genes into Ashbya gossypii.

Attempts have therefore been made, because that [sic] gene from Ashbya gossypii corresponding to the URA3 gene or pyrF gene is unknown, to clone it. Attempts at cloning the Ashbya gene by the methods described in the literature via, for example, hybridization with URA3 gene fragments or via degenerate oligonucleotides based on conserved amino-acid sequences of various orotidine-5'-phosphate decarboxylases and screening a cDNA library using these oligonucleotides and the PCR technique were unsuccessful (Bergkamp et al. Yeast, Vol. 9, 1993: 677 - 681, Piredda et al., Yeast, Vol. 10, 1994: 1601 - 1612, Benito et al., Gene, Vol. 116, 1992: 59 - 67 and Diaz-Minguez et al., Mol. Gen. Genet., Vol. 224, 1990: 269 - 278).

It is an object of the present invention therefore to provide an easily selectable marker which can be transformed with high yield and is easily counterselectable and which makes it possible to insert genes into microorganisms.

We have found that this object is achieved by the novel orotidine-5'-phosphate decarboxylase [lacuna] having the sequence SEQ ID NO: 1 or its homologs which have at least 80% homology with the sequence SEQ ID NO: 1.

Homologs of the novel orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 mean, for example, allelic variants which have at least 80% homology at the derived amino-acid level, preferably at least 90% homology, very particularly preferably at least 95% homology. The amino-acid sequence derived from SEQ ID NO: 1 is to be seen in SEQ ID NO: 1. Allelic variants comprise, in particular, functional variants which are obtainable by deletion, insertion or substitution of nucleotides from the sequence depicted in SEQ ID NO: 1, the intention being, however, that the enzymatic activity of the derived synthesized proteins advantageously be retained for the insertion of one or more genes. However, if the intention is to produce mutants in the orotidine-5'-phosphate decarboxylase gene with the aid of SEQ ID NO: 1 and its homologs in the novel process for producing uracil-auxotrophic microorganisms, non-functional genes will be used, that is to say genes which lead to enzymatically inactive proteins. In this case, it is advantageous to use sequences which display homologies with SEQ ID NO: 1 or its homologs advantageously at the 3' and 5' ends.

Homologs of SEQ ID NO: 1 additionally mean, for example, fungal or plant homologs, truncated sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence. Homologs of SEQ ID NO: 1 have at the DNA level a homology of at least 60%, preferably of at least 70%, particularly preferably of at least 80%, very particularly preferably of at least 90%, over the complete DNA region indicated in SEQ ID NO: 1.

Homologs of SEQ ID NO: 1 also mean derivatives such as, for example, promoter variants. The promoters upstream of the indicated nucleotide sequences may be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without, however, the functionality or activity of the promoters being impaired. It is additionally possible for the promoters to have their activity increased by modifying their sequence, or to be completely replaced by more active promoters even from heterologous organisms.

Derivatives also mean variants whose nucleotide sequence in the region from -1 to -200 in front of the start codon have [sic] been modified so as to alter, preferably increase, gene expression and/or protein expression.

It is possible and preferred for SEQ ID NO: 1 or its homologs to be isolated from microorganisms of the family Metschnikowiaceae, particularly preferably from microorganisms of the genera Eremothecium, Ashbya or Nematospora, very particularly preferably from microorganisms of the genus and species Eremothecium ashbyii or Ashbya gossypii.

The novel gene construct means the URA3 gene sequences [sic] SEQ ID No. 1 and its homologs which have been functionally linked to one or more regulatory signals, advantageously to increase gene expression. Examples of these regulatory sequences are sequences to which inducers or repressors bind and thus regulate the expression of the nucleic acid. In addition to these novel regulatory sequences, the natural regulation of these sequences in front of the actual structural genes can still be present and, where appropriate, have been genetically modified so that the natural regulation has been switched off and the expression of the genes has been increased. The gene construct can, however, also have a simpler structure, that is to say no additional regulatory signals have been inserted in front of the sequence SEQ ID No. 1 or its homologs, and the natural promoter with its regulation has not been deleted. Instead, the natural regulatory sequence has been mutated so that regulation no longer takes place, and gene expression is enhanced. The gene construct may

additionally advantageously comprise one or more so-called enhancer sequences functionally linked to the promoter and making increased expression of the nucleic acid sequence possible. It is also possible to insert at the 3′ end of the DNA sequences additional advantageous sequences, such as further regulatory elements or terminators. The URA3 genes may be present in one or more copies in the gene construct, and the gene or genes can also be inactivated. It is possible with the aid of this or these inactivated genes to generate uracil-auxotrophic mutants in the novel process. It is advantageous for further genes to be present in the gene construct for insertion of further genes into a microorganism. These genes may be located inside a URA3 gene, in which case there ought advantageously to be an intact copy of the URA3 gene and/or another selectable gene such as leu2, thr4 or kan present in the construct, or they can be located outside the URA3 gene. Even if an intact URA3 gene is present in the construct, further markers such as those mentioned above can, where appropriate, be present for selection in the gene construct.

Advantageous regulatory sequences for the novel process are present, for example, in promoters such as cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, $lacI^{q}$, T7, T5, T3, gal, trc, ara, SP6, λ-P_R or λ-P_L promoter and are advantageously used in Gram-negative bacteria. Further advantageous regulatory sequences are present, for example, in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFα , AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S, SSU, OCS, lib4, usp, STLS1, B33, nos or in the ubiquitin or phaseolin promoter. Also advantageous in this connection are the promoters of pyruvate decarboxylase and of methanol oxidase from, for example, Hansenula. It is also possible to use artificial promoters for the regulation.

It is possible in principle to use all natural promoters with their regulatory sequences like those mentioned above for the novel process. It is also possible and advantageous in addition to use synthetic promoters.

The gene construct may, as described above, also comprise further genes which are to be inserted into the microorganisms. These genes can be inserted inside or outside the marker genes such as ura3, leu2, thr4 or kan. It is possible in principle for all types of genes to be inserted into microorganisms with the aid of the novel URA3 gene having the sequence SEQ ID NO: 1 or its homologs. It is possible and advantageous to insert and express in host organisms regulatory genes such as genes for inducers, repressors or enzymes which intervene by their enzymatic activity

in the regulation, or one or more or all genes of a biosynthetic pathway such as the genes of riboflavin biosynthesis such as, for example, the rib genes or genes of biosynthetic pathways which lead to other fine chemicals, secondary metabolites or proteins, such as the genes of biotin, lysine, methionine, vitamin B12 or carotenoid biosynthesis, or genes which lead to flavorings, growth promoters or odoriferous substances, or individual genes for enzymes such as proteases or lipases, via the URA3 sequence. These genes can be heterologous or homologous in origin. The inserted genes may have their own promoter or else be under the control of the promoter of the sequence SEQ ID No. 1 or its homologs.

For expression in the abovementioned host organism, the gene construct is advantageously inserted into a vector such as, for example, a plasmid, a phage or other DNA, which makes optimal expression of the genes in the host possible. Examples of suitable plasmids are, in E. coli, pLG338, pACYC184, pBR322, pUC18, pUC19, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III^{113}-B1, λgt11 or pBdCI, in Streptomyces, pIJ101, pIJ364, pIJ702 or pIJ361, in Bacillus, pUB110, pC194 or pBD214, in Corynebacterium, pSA77 or pAJ667, in fungi, pALS1, pIL2 or pBB116, in yeasts, 2μM, pAG-1, YEp6, YEp13 or pEMBLYe23, or, in plants, pLGV23, pGHlac^{+}, pBIN19, pAK2004 or pDH51. Said plasmids represent a small selection from the possible plasmids. Further plasmids are well known to the skilled worker and can be found, for example, in the book Cloning Vectors (Eds. Pouwels P. H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018).

The gene construct advantageously comprises, for expression of the other genes present, additionally 3′ and/or 5′ terminal regulatory sequences to enhance expression, which are selected for optimal expression depending on the selected host organism and gene or genes.

These regulatory sequences are intended to make specific expression of the genes and of the [sic] protein expression possible. This may mean, depending on the host organism, for example that the gene is expressed or overexpressed only after induction, or that it is immediately expressed and/or overexpressed.

The regulatory sequences or factors may moreover preferably have a beneficial effect on expression of the introduced genes, and thus increase it. It is possible in this way for the regulatory elements to be enhanced advantageously at the transcription level by using strong transcription signals such as promoters and/or

enhancers. However, in addition, it is also possible to enhance translation by, for example, improving the stability of the mRNA.

In a further embodiment of the vector, the novel gene construct can also be advantageously introduced in the form of a linear DNA into the microorganisms and be integrated into the genome of the host organism by heterologous or homologous recombination. This linear DNA can consist of a linearized plasmid or only of the gene construct as vector.

Host organisms suitable in principle for the novel gene construct are all prokaryotic or eukaryotic organisms. The host organisms advantageously used are microorganisms such as bacteria, fungi, yeasts, animal or plant cells. Fungi or yeasts are preferably used, particularly preferably fungi, very particularly preferably fungi of the family Metschnikowiaceae such as Eremothecium, Ashbya or Nematospora.

The invention additionally relates to a process for producing uracil-auxotrophic microorganisms. To generate uracil-auxotrophic mutants, the orotidine-5'-phosphate decarboxylase gene having SEQ ID NO: 1 or its homologs are modified, for example by mutagenesis, in such a way that the protein encoded by the gene is inactivated. This inactivated gene is subsequently introduced into a microorganism, for example by transformation or electroporation. Finally, homologous recombination in the microorganisms results in auxotrophic mutants which can be screened via their resistance to 5-fluoroorotic acid (see Boeke et al., Mol. Gen. Genet., Vol. 197, 1984: 345 - 346).

The invention further relates to a process for inserting DNA into organisms, which comprises inserting into an organism, preferably a microorganism, which is deficient in an orotidine-5'-phosphate decarboxylase gene (= URA3 gene) a vector which comprises an intact URA3 gene having the sequence SEQ ID NO: 1 or its homologs, advantageously together with further DNA, preferably with at least one other gene, and cultivating this organism on or in a culture medium which contains no uracil. Only these organisms which have acquired the vector are able to grow in this medium. A linear DNA is preferably used as vector in this process. The microorganisms preferably used in this process are fungi, especially of the family Metschnikowiaceae such as Eremothecium, Ashbya or Nematosprora [sic], particularly preferably microorganisms of the genus Ashbya.

It is also possible to use as vector any suitable plasmid (but especially a plasmid which harbors the origin of replication of the 2m plasmid from S. cerevisiae) which undergoes autonomous

replication in the cell, but also, as described above, a linear DNA fragment which is integrated into the genome of the host. This integration can take place by heterologous or homologous recombination. But preferably, as mentioned, by homologous recombination (Steiner et al., Genetics, Vol. 140, 1995: 973 - 987).

The novel URA3 gene having the sequence SEQ ID NO: 1 or its homologs can advantageously be used as selection markers in the novel process. It is possible and preferred to insert genes using this selection marker genes [sic] into Ashbya gossypii.

An additional advantage is that on transformation of Ashbya gossypii it is possible to select with the aid of this gene, without the need to use foreign DNA (i.e. DNA not derived from Ashbya gossypii).

It is possible on transformation of Ashbya gossypii with the gene having SEQ ID NO: 1 or its homologs also to insert any other genes. This makes it possible to construct strains which harbor single genes or a plurality of genes in several copies either on plasmids or in the genome.

It is further possible to construct Ashbya strains in which chromosomal copies of genes have been destroyed by the insertion of the URA3 gene having SEQ ID NO: 1 or its homologs.

A particular advantage of the AgURA3 gene is the possibility of using the marker several times in succession in the same strain.

If identical nucleotide sequences are placed 5′ and 3′ of the gene in the same orientation (so-called direct repeats), it is possible if required to delete the AgURA3 marker again by homologous recombination and selection on uracil- and FOA-containing medium, and then in another round insert additional DNA with the aid of this gene. Another advantage is the distinctly greater transformation efficiency by comparison with the markers thr, leu or kan.
In the novel process, the vector comprises as other gene at least one gene of riboflavin synthesis. Genes of riboflavin synthesis mean those genes which are involved in synthesis in the entire metabolism of riboflavin producers such as Ashbya.

Examples:

Example 1:

Production of a genomic gene bank from Ashbya gossypii ATCC10895

Genomic DNA from Ashbya gossypii ATCC10895 was prepared by the process described in WO97/03208. The genomic gene bank derived from this DNA was constructed in pRS314 and in YEp351 (Hill et al., Yeast, Vol. 2, 1986: 163 - 167) by the method described in Sambrook, J. et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press or in [lacuna] F.M. et al. (1994) Current protocols in molecular biology, John Wiley and Sons. As can be inferred from, for example, WO97/03208, other plasmids, such as plasmids of the pRS series (Sikorski and Hieter, Genetics, 1989: 19-27) or cosmids such as, for example, SuperCos1 (Stratagene, La Jolla, USA), are also suitable for producing the gene bank.

Example 2:

It was initially attempted to clone the gene for the orotidine-5'-phosphate decarboxylase (= OMP-DC) from Ashbya gossypii via functional complementation of a corresponding URA3-auxotrophic mutant of Saccharomyces cerevisiae.

To this end, a gene bank was constructed from genomic Ashbya gossypii DNA in pRS314 (as described in Example 1). This DNA was used to transform the S. cerevisiae strain MW3317-21A (genotype: MAT α, trp1, ade8ΔKpn, ura3-52, hom3-10, met13, met4, ade2, his3-Kpn, see, for example, Kramer et al., Mol. Cell. Biol. 9, 1989: 4432-4440), by the lithium acetate method (see, for example, Kramer et al., Mol. Cell. Biol. 9, 1989: 4432-4440). No clone in which the genomic deletion of the ura3 gene of the S. cerevisiae strain was complemented by a gene fragment from Ashbya was obtained.

The attempt to clone the URA3 gene of Ashbya gossypii via functional complementation in a pyrF mutant of E. coli also failed.

Example 3:
An attempt to clone the OMP-DC gene from Ashbya gossypii by hybridization with a fragment of the corresponding gene from Saccharomyces cerevisiae was also unsuccessful.

For this purpose, the complete URA3 gene from Saccharomyces cerevisiae (gene bank entry yscodcd) was used as probe (length 1.1 kb) in order to screen a genomic cosmid gene bank from Ashbya gossypii (see Example 1). The experiment was carried out as described in Sambrook, J. et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press or Ausubel, F.M. et al. (1994) Current protocols in molecular biology, John Wiley and Sons, using hybridization temperatures of 52°C to 68°C. It was not possible to identify in the gene bank any clones which provided a positive signal with the URA3 gene from S. cerevisiae as probe.

Example 4:

In the next approach, it was attempted to clone the gene for OMP-DC from Ashbya gossypii by amplification of gene fragments using degenerate oligonucleotides and the PCR technique.

For this experiment, the known amino-acid sequences of the various orotidine-5'-phosphate decarboxylases from the following organisms were compared, and regions showing maximum conservation in all the enzymes were selected:

Aspergillus niger (Acc. number: P07817)
Aspergillus nidulans (Acc. number: P10652)
Schizosaccharomyces pombe (Acc. number: P14965)
Penicillium chrysogenum (Acc. number: P09463)
Kluyveromyces lactis (Acc. number: P07922)
Candida albicans (Acc. number: P13649)
Neurospora crassa (Acc. number: P05035)
Ustilago maydis (Acc. number: P15188)
Saccharomyces cerevisiae (Acc. number: P03962)
Drosophila melanogaster (Acc. number: Q01637)
Mouse (Acc. number: P13439)
Human (Acc. number: P11172)

The numbers given in parentheses are derived from the SWISS&PIR-Translated Datenbank Release 103.

Degenerate olgonucleotides [sic] were synthesized on the basis of this information.

Degenerate oligonucleotides mean oligonucleotides in which mixtures of nucleotides have been incorporated at several positions during the synthesis.

In this connection, R represents G or A, Y represents C or T, W represents A or T, M represents A or C, K represents G or T, S represents C or G, H represents A, C or T, V represents A, C or G, B represents C, G or T, D represents A, G or T, and N represents G,A,T or C.

The following oligonucleotides were used:

URA3-A: 5'-YTNGGNCCNTAYATHTGY-3'
URA3-B: 5'-TAYTGYTGNCCNARYTTRTCNCC-3«
URA3-C: 5'-TTYYTNATHTTYGARGAYMGNAARTT-3'
URA3-D: 5'-GCNARNARNARNARNCCNC-3'

Using these oligonucleotides as primers, PCRs were carried out with genomic DNA from Ashbya gossypii as template.

The following primer combinations were used:

URA3-A + URA3-B; URA3-A + URA3-D; URA3C + URA3-B and URA3-C + URA3-D.

The following hybridization temperatures were used:

52°C, 48°C, 44°C, 40°C and 37°C.

The products resulting from the PCRs were cloned by conventional methods into the vector pGEMT (Promega) and were sequenced. It was not possible to amplify any fragments which showed homology with the known OMP-DC genes mentioned above.

Example 5:

A cDNA bank was constructed from Ashbya gossypii as described in DE 44 20 785 A1 (Example 1).

Example 6:

Analysis of nucleic acid sequences in the gene bank

Single clones were selected from E.coli clones which comprised the gene bank from Ashbya gossypii described in Example 5. The cells were cultivated by conventional methods in suitable media (e.g. Luria broth with 100 mg/l ampicillin), and plasmid DNA was isolated from these cells.

Oligonucleotides which hybridize in the vector portion were used as primers for sequencing the cDNA clones. Fragments of the cloned cDNAs were detected in this way. The sequences were analyzed as described in Example 7.

Example 7:

A computer-assisted analysis of the nucleotide sequences found was carried out by comparisons of newly identified sequences with existing DNA and protein data banks using the following algorithms, e.g. with BLAST algorithms (Altschul et al. (1990) J. Mol. Biol. 215, 403-410), the Clustal algorithm with the aid of the PAM250 weighting table or the Wilbur-Lipman DNA alignment algorithm (as implemented, for example, in the program package MegAlign 3.06 supplied by DNAstar). It was possible in this way to discover similarities of the newly discovered sequences with previously known sequences, and to describe the function of novel genes or part-sequences of genes.

Example 8:

Identification of E. coli clones which harbor the gene for OMP-DC from Ashbya gossypii (AgURA3).

After examination of a large number of clones as described in Examples 6 and 7 (> 100 clones), a sequence which showed similarities with known OMP-DC genes was found. This homologous process was then used to screen the genomic Ashbya gene bank (see Example 1) once again, and it was possible to identify clones and cosmids which gave a specific positive signal and harbored a common 1.3 kb XhoI-EcoRI fragment. Sequencing of the clones produced the sequence as described in SEQ ID NO: 1. The sequence shows similarities with known URA3 genes and codes for a protein about 29246 Dalton in size.

Example 9:

Disruption of the chromosomal copy of the AgURA3 gene with antibiotic resistance genes

Disruption of a gene means destruction of the functionality of a genomic copy of the gene either by (a) deleting part of the gene sequence or by (b) of the [sic] interrupting the gene by introducing a piece of foreign DNA into the gene or by (c) replacing part of the gene by foreign DNA. Any foreign DNA can be used, but it is preferably a gene which effects resistance to any

suitable chemical. Any suitable resistance genes can be used to disrupt genes.

To disrupt the AgURA3 gene of Ashbya gossypii ATCC10895, the kanamycin resistance gene from Tn903, which [lacuna] under the control of the TEF promoter of Ashbya gossypii (see Yeast 10, pages 1793-1808, 1994 or WO92/00379), was used. The gene is flanked 5′ and 3′ by several cleavage sites for restriction endonucleases, so that it was possible to construct a cassette which make [sic] possible any desired constructions of gene disruptions using conventional methods of *in vitro* DNA manipulation.

The internal 370 bp PstI-KpnI fragment of AgURA3 (position 442 - 892 in sequence SEQ ID NO: 1) was replaced by a resistance cassette as outline above. The resulting construct was given the name ura3::G418. The resulting plasmid can be replicated after transformation into E.coli. The XhoI-SphI fragment of the construct ura3::G418 (see Figure 1) was purified by agarose gel electrophoresis and subsequent elution of the DNA from the gel (see Proc. Natl. Acad. Sci. USA 76, 615-619, 1979) and employed to transform Ashbya gossypii. Figure 1 shows in depiction A a restriction map of the coding region of the AgURA3 gene and of the 5′- and 3′-untranslated regions (= 5′-UTR and 3′-UTR). Depiction B shows the situation after insertion of the kanamycin resistance cassette described above (= TEF-kanR).

The fragment was transformed into Ashbya gossypii either by protoplast transformation (Gene 109, 99-105, 1991) or else, preferably, by electroporation (BioRad Gene Pulser, conditions: cuvettes with slit widths of 0.4 mm, 1500V, 25μF, 100Ω). The selection of transformed cells took place on G418-containing solid medium (WO 97/03208).

Resulting G418-resistant clones were examined by conventional methods of PCR and Southern blot analysis (Sambrook, J. et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press and in [lacuna] F.M. et al. (1994) Current protocols in molecular biology, John Wiley and Sons) to find whether the genomic copy of the AgURA3 gene was in fact destroyed. Clones whose AgURA3 gene was destroyed are uracil-auxotrophic and resistant to 1 mg/ml 5′-fluoroorotic acid (FOA).

Example 10:

Disruption of the chromosomal copy of the AgURA3 gene without using antibiotic resistance genes

A particular advantage of the use of URA3 genes is the possibility of selection both for the presence and for the absence of the gene. It is possible to screen with FOA microorganisms which have a functionally inactivated URA3 gene, and by means of selection for uracil prototrophy to select for a functionally active URA3 gene.

To disrupt the genomic copy of the URA3 gene, for the sake of simplicity an internal fragment (= PstI fragment) of the URA3 gene was deleted from the coding region of the gene having the sequence SEQ ID NO: 1 (position 442 to 520 in sequence SEQ ID NO: 1). Transformation of Ashbya gossypii with this deleted ura3 fragment was carried out as described in Example 10. In place of deletion of part-regions of the gene, it is also possible in principle to use all other methods for inactivating the gene, such as mutations via insertions, duplications, reversions, replacement of several nucleotides or point mutations. Point mutations are less preferred because reversion thereof is easy.

The transformants were selected through resistance to FOA. In contrast to wild-type clones, clones which harbor a disruption of the AgURA3 gene are resistant to 1 mg/ml FOA.

Example 11:

Use of the AgURA3 gene for inserting further DNA into A. gossypii.

The isocitrate lyase gene described in WO 97/03208 was inserted with the aid of the plasmid pAG100, as described in WO 97/03208 (Example 4 and 5), into AgURA3 disruption mutants of A. gossypii (see Example 9 and 10), using as selection marker in A. gossypii the AgURA3 gene in place of the G418 resistance described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: BASF Aktiengesellschaft
(B) STREET: Carl Bosch Strasse
(C) CITY: Ludwigshafen
(D) FEDERAL STATE: Rheinland-Pfalz
(E) COUNTRY: Germany
(F) POSTAL CODE: D-67056

(ii) TITLE OF APPLICATION: Orotidine-5'-phosphate decarboxylase gene, gene construct comprising this gene and its use

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER-READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1380 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) [sic] ANTISENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Ashbya gossypii

(vii) IMMEDIATE SOURCE:
(B) CLONE: ura3

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 210..1013

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..199

(ix) FEATURES:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1014..1380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
CTCGAGCAAC TCATTGGAAG CCCTTCGCAA ACGACCTCTA TATCTCGTCT CAAGTTCCTA      60

CTATCATGTA TGCTGTCACT ACAGAAAAAT TTTTGTCTAT AGCTGGCAAG AAGCACATCA     120

CATACATTCT GATGGTGTAG GCTCCACATC ACAGTAAGCA TTTGTATAAG GCTGATCACA     180

TAGGGTGCTA CCGACCTAGC CATTGCCAC ATG TCA ACG AAA TCT TAC GCA GAA       233
                                Met Ser Thr Lys Ser Tyr Ala Glu
                                 1               5

AGG GCC AAG GCA CAC AAT TCG CCA GTT GCT AGA AAG CTT CTG GCA TTG       281
Arg Ala Lys Ala His Asn Ser Pro Val Ala Arg Lys Leu Leu Ala Leu
     10                  15                  20

ATG CAC GAG AAG AAA ACC AAT CTC TGC GCT TCC CTT GAT GTG CGG ACG       329
Met His Glu Lys Lys Thr Asn Leu Cys Ala Ser Leu Asp Val Arg Thr
 25                  30                  35                  40

TCT AGA AAG CTT CTG GAG CTA GCA GAC ACG CTG GGA CCG CAC ATT TGT       377
Ser Arg Lys Leu Leu Glu Leu Ala Asp Thr Leu Gly Pro His Ile Cys
                 45                  50                  55

CTG CTG AAG ACA CAT GTC GAC ATA CTG ACG GAC TTC GAC ATC GAG ACG       425
Leu Leu Lys Thr His Val Asp Ile Leu Thr Asp Phe Asp Ile Glu Thr
             60                  65                  70

ACA GTC AAG CCG CTG CAG CAG CTT GCG GCT AAG CAC AAC TTC ATG ATC       473
Thr Val Lys Pro Leu Gln Gln Leu Ala Ala Lys His Asn Phe Met Ile
         75                  80                  85

TTC GAG GAC CGC AAG TTC GCT GAC ATT GGC AAC ACG GTT AAG CTG CAG       521
Phe Glu Asp Arg Lys Phe Ala Asp Ile Gly Asn Thr Val Lys Leu Gln
     90                  95                  100

TAC TCC TCC GGC GTG TAC CGT ATC GCG GAG TGG GCG GAT ATT ACC AAT       569
Tyr Ser Ser Gly Val Tyr Arg Ile Ala Glu Trp Ala Asp Ile Thr Asn
105                 110                 115                 120
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GCA CAC GGC GTC ACC GGC CCC GGT GTG ATA GCC GGG CTG AAG GAG GCT      617
Ala His Gly Val Thr Gly Pro Gly Val Ile Ala Gly Leu Lys Glu Ala
                125                 130                 135

GCG AAA CTG GCC TCA CAG GAA CCC AGG GGG TTG CTG ATG CTG GCA GAG      665
Ala Lys Leu Ala Ser Gln Glu Pro Arg Gly Leu Leu Met Leu Ala Glu
            140                 145                 150

CTC TCT TCT CAG GGC TCT TTG GCG CGC GGA GAC TAT ACC GCG GGC GTC      713
Leu Ser Ser Gln Gly Ser Leu Ala Arg Gly Asp Tyr Thr Ala Gly Val
        155                 160                 165

GTT GAA ATG GCG AAG CTG GAC GAA GAC TTT GTG ATC GGG TTC ATC GCG      761
Val Glu Met Ala Lys Leu Asp Glu Asp Phe Val Ile Gly Phe Ile Ala
    170                 175                 180

CAG CGT GAC ATG GGT GGG CGT GCA GAC GGC TTT GAC TGG CTC ATC ATG      809
Gln Arg Asp Met Gly Gly Arg Ala Asp Gly Phe Asp Trp Leu Ile Met
185                 190                 195                 200

ACC CCG GGG GTT GGC CTG GAC GAC AAA GGA GAC GGC CTG GGC CAG CAG      857
Thr Pro Gly Val Gly Leu Asp Asp Lys Gly Asp Gly Leu Gly Gln Gln
                205                 210                 215

TAC CGC ACG GTG GAT GAG GTC GTC AGC GAC GGT ACC GAT GTG ATC ATT      905
Tyr Arg Thr Val Asp Glu Val Val Ser Asp Gly Thr Asp Val Ile Ile
            220                 225                 230

GTT GGC AGA GGG CTC TTT GAC AAG GGA AGA GAC CCC AAG GTC GAG GGT      953
Val Gly Arg Gly Leu Phe Asp Lys Gly Arg Asp Pro Lys Val Glu Gly
        235                 240                 245

GCC CGC TAC CGC AAG GCC GGT TGG GAG GCT TAC TTG CGC CGT ATG GGC     1001
Ala Arg Tyr Arg Lys Ala Gly Trp Glu Ala Tyr Leu Arg Arg Met Gly
    250                 255                 260

GAG ACT TCG TAGTCTATCG CTGGCGCCCA CAGTATATAG GCGGATTCCA             1050
Glu Thr Ser
265

CCGCCGATTA CCATCTCAGC AACCTTTTTG TAATTATATG CCCCTATTGC CCTTATTTCC   1110

GAGCTGGTGC CGGGATCGGT TTATAGACGG GCAACAAGTT GATACTTTGT TCAGTAGCAT   1170

GCATCCAACA CTTGCAGGCT TGGGGTGTGG AAGGCCTCGC CGCGGATAAT TCGTATTACC   1230

CGCACTTCGT GAAGTATTGC TTTATGAAAA ATCTTCACTT TGGGCTAACT AGAGCCATAA   1290

CTCGACACAA GCCCCTTCCT ACACACTTCG AGCTGGGACT AAAGTGACAA CGAATAGCAA   1350
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ATAATTAGCA AATATGGATG CGTTGAATTC 1380

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ser Thr Lys Ser Tyr Ala Glu Arg Ala Lys Ala His Asn Ser Pro
  1               5                  10                  15

Val Ala Arg Lys Leu Leu Ala Leu Met His Glu Lys Lys Thr Asn Leu
             20                  25                  30

Cys Ala Ser Leu Asp Val Arg Thr Ser Arg Lys Leu Leu Glu Leu Ala
         35                  40                  45

Asp Thr Leu Gly Pro His Ile Cys Leu Leu Lys Thr His Val Asp Ile
     50                  55                  60

Leu Thr Asp Phe Asp Ile Glu Thr Thr Val Lys Pro Leu Gln Gln Leu
 65                  70                  75                      80

Ala Ala Lys His Asn Phe Met Ile Phe Glu Asp Arg Lys Phe Ala Asp
                 85                  90                  95

Ile Gly Asn Thr Val Lys Leu Gln Tyr Ser Ser Gly Val Tyr Arg Ile
            100                 105                 110

Ala Glu Trp Ala Asp Ile Thr Asn Ala His Gly Val Thr Gly Pro Gly
        115                 120                 125

Val Ile Ala Gly Leu Lys Glu Ala Ala Lys Leu Ala Ser Gln Glu Pro
    130                 135                 140

Arg Gly Leu Leu Met Leu Ala Glu Leu Ser Ser Gln Gly Ser Leu Ala
145                 150                 155                     160

Arg Gly Asp Tyr Thr Ala Gly Val Val Glu Met Ala Lys Leu Asp Glu
                165                 170                 175

Asp Phe Val Ile Gly Phe Ile Ala Gln Arg Asp Met Gly Gly Arg Ala
            180                 185                 190
```

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Asp Gly Phe Asp Trp Leu Ile Met Thr Pro Gly Val Gly Leu Asp Asp
        195                 200                 205

Lys Gly Asp Gly Leu Gly Gln Gln Tyr Arg Thr Val Asp Glu Val Val
    210                 215                 220

Ser Asp Gly Thr Asp Val Ile Ile Val Gly Arg Gly Leu Phe Asp Lys
225                 230                 235                 240

Gly Arg Asp Pro Lys Val Glu Gly Ala Arg Tyr Arg Lys Ala Gly Trp
                245                 250                 255

Glu Ala Tyr Leu Arg Arg Met Gly Glu Thr Ser
            260                 265
```

Orotidine-5′-phosphate decarboxylase gene, gene construct comprising this gene and its use

Abstract

The invention relates to an orotidine-5′-phosphate decarboxylase gene having the sequence SEQ ID No. 1 or its homologs, to a gene construct comprising this gene or its homologs, and to its use. The invention additionally relates to vectors or organisms comprising an orotidine-5′-phosphate decarboxylase gene having the sequence SEQ ID No. 1 or its homologs.

The invention further relates to a process for producing uracil-auxotrophic microorganisms and to a process for inserting DNA into uracil-auxotrophic microorganisms.

SEQUENZPROTOKOLL

(1) ALGEMEINE INFORMATION:

(i) ANMELDER:
(A) NAME: BASF Aktiengesellschaft
(B) STRASSE: Carl Bosch Strasse
(C) ORT: Ludwigshafen
(D) BUNDESLAND: Rheinland-Pfalz
(E) LAND: Germany
(F) POSTLEITZAHL: D-67056

(ii) ANMELDETITEL: Orotidin-5'-Phosphatdecarboxylase-Gen, Genkonstrukt enthaltend dieses Gen und seine Verwendung

(iii) ANZAHL DER SEQUENZEN: 2

(iv) COMPUTER-LESBARE FORM:
(A) DATENTRÄGER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) BETRIEBSSYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPA)

(2) INFORMATION ZU SEQ ID NO: 1:

(i) SEQUENZ CHARAKTERISTIKA:
(A) LÄNGE: 1380 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: DNS (genomisch)

(iii) HYPOTHETISCH: NEIN

(iii) ANTISENSE: NEIN

(vi) URSPRÜNLICHE HERKUNFT:
(A) ORGANISMUS: Ashbya gossypii

(vii) UNMITTELBARE HERKUNFT:
(B) CLON: ura3

(ix) MERKMALE:
(A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 210..1013

(ix) MERKMALE:
(A) NAME/SCHLÜSSEL: 5'UTR
(B) LAGE: 1..199

```
(ix) MERKMALE:
      (A) NAME/SCHLÜSSEL: 3'UTR
      (B) LAGE: 1014..1380

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 1:

CTCGAGCAAC TCATTGGAAG CCCTTCGCAA ACGACCTCTA TATCTCGTCT CAAGTTCCTA      60

CTATCATGTA TGCTGTCACT ACAGAAAAAT TTTTGTCTAT AGCTGGCAAG AAGCACATCA     120

CATACATTCT GATGGTGTAG GCTCCACATC ACAGTAAGCA TTTGTATAAG GCTGATCACA     180

TAGGGTGCTA CCGACCTAGC CATTGCCAC ATG TCA ACG AAA TCT TAC GCA GAA      233
                                Met Ser Thr Lys Ser Tyr Ala Glu
                                 1               5

AGG GCC AAG GCA CAC AAT TCG CCA GTT GCT AGA AAG CTT CTG GCA TTG      281
Arg Ala Lys Ala His Asn Ser Pro Val Ala Arg Lys Leu Leu Ala Leu
     10                  15                  20

ATG CAC GAG AAG AAA ACC AAT CTC TGC GCT TCC CTT GAT GTG CGG ACG      329
Met His Glu Lys Lys Thr Asn Leu Cys Ala Ser Leu Asp Val Arg Thr
 25                  30                  35                  40

TCT AGA AAG CTT CTG GAG CTA GCA GAC ACG CTG GGA CCG CAC ATT TGT      377
Ser Arg Lys Leu Leu Glu Leu Ala Asp Thr Leu Gly Pro His Ile Cys
                 45                  50                  55

CTG CTG AAG ACA CAT GTC GAC ATA CTG ACG GAC TTC GAC ATC GAG ACG      425
Leu Leu Lys Thr His Val Asp Ile Leu Thr Asp Phe Asp Ile Glu Thr
             60                  65                  70

ACA GTC AAG CCG CTG CAG CAG CTT GCG GCT AAG CAC AAC TTC ATG ATC      473
Thr Val Lys Pro Leu Gln Gln Leu Ala Ala Lys His Asn Phe Met Ile
         75                  80                  85

TTC GAG GAC CGC AAG TTC GCT GAC ATT GGC AAC ACG GTT AAG CTG CAG      521
Phe Glu Asp Arg Lys Phe Ala Asp Ile Gly Asn Thr Val Lys Leu Gln
     90                  95                 100

TAC TCC TCC GGC GTG TAC CGT ATC GCG GAG TGG GCG GAT ATT ACC AAT      569
Tyr Ser Ser Gly Val Tyr Arg Ile Ala Glu Trp Ala Asp Ile Thr Asn
105                 110                 115                 120

GCA CAC GGC GTC ACC GGC CCC GGT GTG ATA GCC GGG CTG AAG GAG GCT      617
Ala His Gly Val Thr Gly Pro Gly Val Ile Ala Gly Leu Lys Glu Ala
                125                 130                 135

GCG AAA CTG GCC TCA CAG GAA CCC AGG GGG TTG CTG ATG CTG GCA GAG      665
Ala Lys Leu Ala Ser Gln Glu Pro Arg Gly Leu Leu Met Leu Ala Glu
            140                 145                 150
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Leu Ser Ser Gln Gly Ser Leu Ala Arg Gly Asp Tyr Thr Ala Gly Val
155 160 165

GTT GAA ATG GCG AAG CTG GAC GAA GAC TTT GTG ATC GGG TTC ATC GCG 761
Val Glu Met Ala Lys Leu Asp Glu Asp Phe Val Ile Gly Phe Ile Ala
170 175 180

CAG CGT GAC ATG GGT GGG CGT GCA GAC GGC TTT GAC TGG CTC ATC ATG 809
Gln Arg Asp Met Gly Gly Arg Ala Asp Gly Phe Asp Trp Leu Ile Met
185 190 195 200

ACC CCG GGG GTT GGC CTG GAC GAC AAA GGA GAC GGC CTG GGC CAG CAG 857
Thr Pro Gly Val Gly Leu Asp Asp Lys Gly Asp Gly Leu Gly Gln Gln
205 210 215

TAC CGC ACG GTG GAT GAG GTC GTC AGC GAC GGT ACC GAT GTG ATC ATT 905
Tyr Arg Thr Val Asp Glu Val Val Ser Asp Gly Thr Asp Val Ile Ile
220 225 230

GTT GGC AGA GGG CTC TTT GAC AAG GGA AGA GAC CCC AAG GTC GAG GGT 953
Val Gly Arg Gly Leu Phe Asp Lys Gly Arg Asp Pro Lys Val Glu Gly
235 240 245

GCC CGC TAC CGC AAG GCC GGT TGG GAG GCT TAC TTG CGC CGT ATG GGC 1001
Ala Arg Tyr Arg Lys Ala Gly Trp Glu Ala Tyr Leu Arg Arg Met Gly
250 255 260

GAG ACT TCG TAGTCTATCG CTGGCGCCCA CAGTATATAG GCGGATTCCA 1050
Glu Thr Ser
265

CCGCCGATTA CCATCTCAGC AACCTTTTTG TAATTATATG CCCCTATTGC CCTTATTTCC 1110

GAGCTGGTGC CGGGATCGGT TTATAGACGG GCAACAAGTT GATACTTTGT TCAGTAGCAT 1170

GCATCCAACA CTTGCAGGCT TGGGGTGTGG AAGGCCTCGC CGCGGATAAT TCGTATTACC 1230

CGCACTTCGT GAAGTATTGC TTTATGAAAA ATCTTCACTT TGGGCTAACT AGAGCCATAA 1290

CTCGACACAA GCCCCTTCCT ACACACTTCG AGCTGGGACT AAAGTGACAA CGAATAGCAA 1350

ATAATTAGCA AATATGGATG CGTTGAATTC 1380

(2) INFORMATION ZU SEQ ID NO: 2:

(i) SEQUENZ CHARAKTERISTIKA:
(A) LÄNGE: 267 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 2:

```
Met Ser Thr Lys Ser Tyr Ala Glu Arg Ala Lys Ala His Asn Ser Pro
 1               5                  10                  15

Val Ala Arg Lys Leu Leu Ala Leu Met His Glu Lys Lys Thr Asn Leu
            20                  25                  30

Cys Ala Ser Leu Asp Val Arg Thr Ser Arg Lys Leu Leu Glu Leu Ala
        35                  40                  45

Asp Thr Leu Gly Pro His Ile Cys Leu Leu Lys Thr His Val Asp Ile
    50                  55                  60

Leu Thr Asp Phe Asp Ile Glu Thr Thr Val Lys Pro Leu Gln Gln Leu
65                  70                  75                  80

Ala Ala Lys His Asn Phe Met Ile Phe Glu Asp Arg Lys Phe Ala Asp
                85                  90                  95

Ile Gly Asn Thr Val Lys Leu Gln Tyr Ser Ser Gly Val Tyr Arg Ile
            100                 105                 110

Ala Glu Trp Ala Asp Ile Thr Asn Ala His Gly Val Thr Gly Pro Gly
        115                 120                 125

Val Ile Ala Gly Leu Lys Glu Ala Ala Lys Leu Ala Ser Gln Glu Pro
    130                 135                 140

Arg Gly Leu Leu Met Leu Ala Glu Leu Ser Ser Gln Gly Ser Leu Ala
145                 150                 155                 160

Arg Gly Asp Tyr Thr Ala Gly Val Val Glu Met Ala Lys Leu Asp Glu
                165                 170                 175

Asp Phe Val Ile Gly Phe Ile Ala Gln Arg Asp Met Gly Gly Arg Ala
            180                 185                 190

Asp Gly Phe Asp Trp Leu Ile Met Thr Pro Gly Val Gly Leu Asp Asp
        195                 200                 205

Lys Gly Asp Gly Leu Gly Gln Gln Tyr Arg Thr Val Asp Glu Val Val
    210                 215                 220

Ser Asp Gly Thr Asp Val Ile Ile Val Gly Arg Gly Leu Phe Asp Lys
225                 230                 235                 240

Gly Arg Asp Pro Lys Val Glu Gly Ala Arg Tyr Arg Lys Ala Gly Trp
                245                 250                 255

Glu Ala Tyr Leu Arg Arg Met Gly Glu Thr Ser
            260                 265
```

ART 34 AMDT 09/582779

534 Rec'd PCT/PTO 03 JUL 2000

We claim:

1. An orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 or its homologs which have at least 80% homology with the sequence SEQ ID NO: 1.

2. An orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 or its homologs, wherein the gene or its homologs derive from Ashbya gossypii.

3. An amino-acid sequence encoded by a gene or its homologs as claimed in claim 1 or 2.

4. An amino-acid sequence as claimed in claim 3, which comprises an enzymatically active protein.

5. A gene construct comprising an orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 or its homologs as claimed in claim 1 or 2, where the gene or its homologs is functionally linked to one or more regulatory signals.

6. A gene construct as claimed in claim 5, whose gene expression is increased by the regulatory signals.

7. A vector comprising a gene construct as claimed in claim 5 or 6.

8. A microorganism comprising at least one gene construct as claimed in claim 5 or 6.

9. A process for producing uracil-auxotrophic microorganisms, which comprises modifying an orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 or its homologs as claimed in claim 1 or 2 in such a way that the protein encoded by the gene is inactive, and this modified gene is introduced into the microorganisms and integrated by homologous recombination into the genome of the organisms, and subsequently these microorganisms are selected for resistance to 5-fluoroorotic acid.

10. A process for inserting DNA into microorganisms, which comprises inserting a vector which comprises an intact orotidine-5'-phosphate decarboxylase gene having the sequence SEQ ID NO: 1 or its homologs as claimed in claim 1 or 2, together with at least one other gene, into a microorganism

ART 34 AMDT

which is deficient in orotidine-5'-phosphate decarboxylase genes, and cultivating this microorganism on or in a culture medium without uracil.

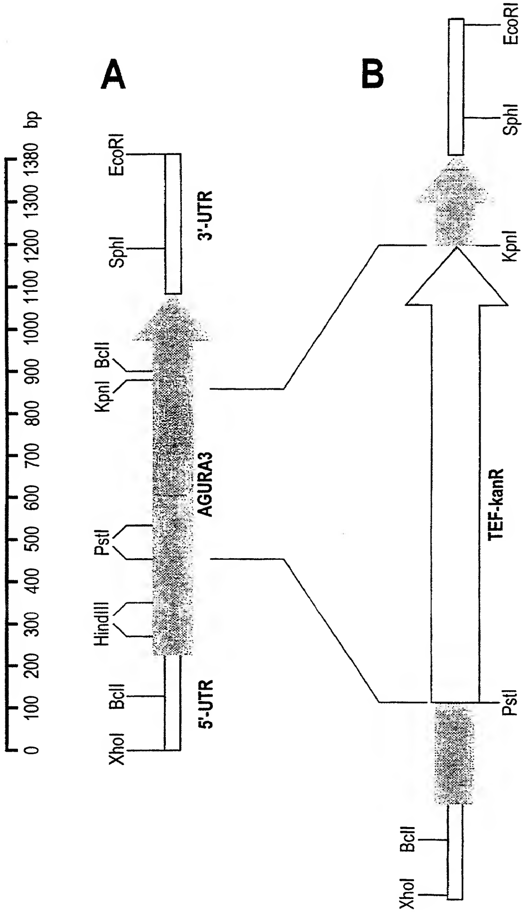
11. A process as claimed in claim 10, wherein a linear DNA is used as vector.

12. A process as claimed in claim 10 or 11, wherein an Ashbya gossypii strain is used as microorganism deficient in orotidine-5'-phosphate decarboxylase genes.

13. A process as claimed in any of claims 10 to 12, wherein at least one gene of riboflavin synthesis is inserted as other gene into the microorganism.

14. The use of a gene sequence or its homologs as claimed in claim 1 or 2 as selection marker.

15. The use as claimed in claim 14 in Ashbya gossypii.


A
B
bp
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1380
XhoI
BclI
HindIII
PstI
KpnI
BclI
SphI
EcoRI
5'-UTR
AGURA3
3'-UTR
XhoI
BclI
PstI
TEF-kanR
KpnI
SphI
EcoRI

Declaration, Power of Attorney

0050/048715

We (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Orotidine-5'-phosphate decarboxylase gene, gene construct comprising this gene and its use

the specification of which

[x] is attached hereto.

[ ] was filed on ______________________ as

Application Serial No. ______________________

and amended on ______________________.

[x] was filed as PCT international application

Number PCT/EP 98/08382

on 18/12/1998

and was amended under PCT Article 19

on ______________________ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above–identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)–(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
19801120.2	*Germany*	*15 January 1998*	[x] Yes [ ] No

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We (I) hereby claim the benefit under Title 35, United States Codes, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

09/763950

Application Serial No.	**Filing Date**	**Status (pending, patented, abandoned)**

2 And we (I) hereby appoint **Messrs. HERBERT. B. KEIL**, Registration Number 18,967; and **RUSSEL E. WEINKAUF**, Registration Number 18,495; the address of both being Messrs. Keil & Weinkauf, 1101 Connecticut Ave., N.W., Washington, D.C. 20036 (telephone 202-659-0100), our attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to sign the drawings, to receive the patent, and to transact all business in the Patent Office connected therewith.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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